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Laser-assisted microdissection of plant embryos for transcriptional profiling

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Running Head: Embryo laser-assisted microdissection

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Abstract

Transcriptomic studies have proven powerful and effective as a tool to study the molecular underpinnings of plant development. Still, it remains challenging to disentangle cell-or tissue-specific transcriptomes in complex structures like the plant seed. In particular, the embryo of flowering plants is embedded in the endosperm, a nurturing tissue, which in turn is enclosed by the maternal seed coat. Here we describe laser-assisted microdissection (LAM) to isolate highly pure embryo tissue from whole seeds. This technique is applicable to virtually any plant seed, and we illustrate the use of LAM to isolate embryos from species of the *Boechera* and *Solanum* genera. LAM is a tool that will greatly help to increase the repertoires of tissue-specific transcriptomes, including those of embryos and parts thereof, in non-model plants.

Keywords

Boechera, embryo, laser-assisted microdissection, RNA-Seq, *Solanum*, tissue-specificity, transcriptome

1 Introduction

Studies of the transcriptional basis of plant embryogenesis have so far been largely restricted to the model plant *Arabidopsis thaliana* *(1-12)*, but some analyses have also been performed in maize *(13-15)* and rice *(16, 17)*. Most of these studies described expression patterns in different regions of the embryo or at different stages of embryogenesis *(1-7, 13, 14, 16)* or focused on the regulation and dynamics of allele-specific parental contributions to gene expression in the early embryo *(8-12, 15, 17)*. The seed is composed of tissues that differ in ploidy as well as parental contributions *(18, 19)*. Thus, to properly study the embryonic transcriptome, the embryo must be removed from its accompanying tissues in the seed, the endosperm and the seed coat. Methods that involved manual dissection of embryos from their surrounding tissues have been developed *(20)* and variations thereof are widely used *(e.g. 4, 5)*. Other methods involve the use of transgenic lines allowing the profiling of individual cell and tissue types, such as fluorescence-activated cell or nuclei sorting (FACS, FANS) *(3, 21, 22)*. Labelling and affinity purification of nuclei (INTACT) *(23, 24)* or polysomes (TRAP) *(25, 26)* also allow the analysis of gene expression at a finer scale. Because all these methods require the expression of transgenes under highly specific embryonic promoters, their use has so far largely been restricted to *Arabidopsis*.

Concerns have been raised about potential contamination of embryonic samples with debris from surrounding maternal tissues *(27)*. Given that many genes are expressed in both embryos and seed coat *(28)*, it is not easy to ascertain contamination without independent confirmation of tissue-specific expression. Nonetheless, many transcriptome datasets obtained from manually dissected embryos or embryonic nuclei recovered by FANS detected genes that were also found expressed in the seed coat while embryo and endosperm datasets obtained by LAM showed less expression of such genes *(27)*. This

highlights that, in comparison to other methods, LAM allows the isolation of very precise and highly pure samples for transcriptome studies of reproductive tissues *(29-32)*. Another important advantage of LAM is that, in contrast to other methods allowing the profiling of individual cell and tissue types such as INTACT and TRAP, LAM does not require the generation of transgenic marker lines for cell capture. This makes LAM applicable to any plant embryo or tissue of interest without prior molecular knowledge and is thus a powerful method to study non-model organisms. Here we present an LAM methodology adapted to plant embryos. We have implemented this protocol in species of the genus *Boechera,* a member of the Brassicasea closely related to *Arabidopsis*, and in wild tomatoes, belonging to the genus *Solanum*, in order to exemplify the versatility and broad applicability of LAM for obtaining transcriptomic data of high purity and quality from virtually any plant embryo.

2 Materials

2.1 Removal of RNAses from working material and equipment

1. RNaseZAP™ R2020 Sigma
2. RNase-free water
3. Ethanol 70%

2.2 Tissue collection and fixation

1. *Boechera* and wild tomato plants with fruits and siliques at the desired stage.
2. Farmer's fixation solution: Ethanol 90% (v/v) acetic acid 10% (v/v).
3. Microcentrifuge tubes
4. Ice and insulated box
5. Forceps
6. Scissors
7. Double-sided tape
8. Glass slides
9. Razor blade
10. Excicator/ vacuum chamber

2.3 Tissue embedding, blocking, and slide preparation

1. Tissue-Loc™ HistoScreen™ Cassettes - Thermo Scientific™
2. Pencil
3. Ice and insulated box
4. RNase-free water
5. 70% (v/v) ethanol (EtOH)
6. Large glass staining container
7. Embedding machine, HistoCore Pearl - Leica GmbH (Wetzlar, Germany)
8. Blocking station, HistoCore Arcadia H EG1150C - Leica GmbH (Wetzlar, Germany)
9. Flat bottom plastic weighing dish
10. Preparation needle
11. Forceps
12. Spatula
13. Razor blade

14. Sample holder for microtome
15. Paraffin
16. Bottom illuminated table
17. Ethanol torch
18. Rotary microtome RM2255 - Leica GmbH (Wetzlar, Germany)
19. Microtome steel blades
20. Black cardboard
21. LCM-slides: nuclease-free PET membrane slides 1.4µm - MicroDissect GmbH
22. Heating plate
23. Sterile pipette
24. Staining dish
25. Histoclear™ or Xylol

2.4 Laser-assisted microdissection (LAM)

1. DUST OFF 67, compressed air spray
2. Laser capture microscope (LCM) Cellcut IX71, consisting of an Olympus IX81 inverted microscope with an Olympus light box IX2-UCB.
3. MMI Cellcut® laser - MMI AG (Glattbrugg, Switzerland)
4. Software package MMI CellTools version 4.4.9. - MMI AG (Glattbrugg, Switzerland)
5. MMI Isolation caps transparent 0.5 mL Prod. No. 50204
6. Glass slides for microscopy

3 Methods

3.1 Removal of RNAses from working material and equipment

1. To ensure an RNAse free environment, bake any glassware for ~ 8 hr at 180 °C.
2. Treat any working material and equipment to be used with RNAse decontamination solution. Remove the decontamination solution with RNAse-free water and, subsequently, clean the surfaces further with 70% EtOH.

3.2 Tissue collection and fixation

1. Prepare microcentrifuge tubes filled with farmer's fixation solution on ice (*see* Note 1).
2. Remove the tissue (wild tomato fruits or developing *Boechera* siliques) containing embryos at the targeted developmental stage with forceps and scissors (*see* Note 2).
3. For *Boechera* embryos, place the silique on a glass slice with double-sided tape and carefully cut open the valves along the replum *(33)* (*see* Note 3). For wild tomato embryos cut the fruits in half with a razor blade.
4. Immerse the tissue rapidly in the fixation solution on the microcentrifuge tube placed on ice.
5. Place the opened tubes with the samples on an excicator filled with ice and vacuum infiltrate for 30 minutes with a release after 15 min.
6. Store overnight at 4°C.

3.3 Tissue embedding, blocking, and slide preparation.

1. Transfer fixed tissue to ice-cold, nuclease-free 70% EtOH. Proceed to embedding on the same day.
2. Prepare a large staining container glass filled with ice-cold, nuclease-free 70% EtOH that accommodates all samples to process. Transfer tissue to embedding cassettes, firmly close the cassettes, and label them appropriately with a pencil. Deposit the cassettes with the samples in the ice-cold ethanol glass container. Work fast to avoid drying of the tissue (*see* Note 4).
3. Transfer cassettes to sample basket and run embedding machine overnight following the manufacturer's instructions. Recommended standard settings are 1 hr 70% EtOH, three times 1 hr 90% EtOH, three times 1 hr 100% EtOH, two times 1 hr 100% Xylol, one time 1 hr 15 min 100% Xylol at room temperature, followed by infiltration with paraffin wax two times for 1 hr and one time for 3 hr at 56 °C.
4. Set blocking station to pre-heat several hours before the planned start of use.
5. Transfer the sample basket to the pre-heated blocking station.
6. Using the blocking station, fill a plastic weighing dish with paraffin wax at 56 °C. Lift the lid of the paraffin bath, pick up only one cassette at a time, and transfer the samples from the cassettes to the liquid paraffin wax at 56 °C in the balancing tray using a pair of tweezers. Work fast to avoid solidification of the wax around the samples before placing them in the weighing dishes (*see* Note 5).
7. Position samples well interspaced among each other and in a position that maximizes embryo recovery when preparing thin sections (*see* Note 6).
8. After letting the paraffin completely solidify at room temperature, store at 4 °C until further processing.
9. Remove paraffin block from weighing dish, place on a bottom illuminated table, and cut the paraffin block into pieces containing the target sample.
10. Coat the grid surface of labeled sample holder by adding melted paraffin using a spatula and ethanol torch. While the paraffin is still soft, adhere the previously prepared block of paraffin with samples onto the sample holder.
11. Trim the edges of the paraffin block and take care to maintain parallel edges in order to have straight ribbons when preparing thin sections (*see* Note 7). Before proceeding with sectioning, place samples at 4°C to harden paraffin for at least 10 min.
12. Set heating plate to 42°C
13. Safely clamp-in the sample holder into a microtome equipped with a fresh microtome steel blade. Set the microtome to 7-8 µm and cut the block into ribbons (*see* Note 8). Refer to manufacturer's instructions for using the microtome. Place the ribbons onto a box with black cardboard using brush and forceps.
14. Using the brush, forceps, and curved surgical blade, divide the ribbons into pieces containing embryos. Observe ribbons under stereoscope if needed to identify the desired samples.
15. Place an adequate number of LCM slides onto the hotplate (42°C) with the flat surface covered by a PET membrane facing up.
16. Using the pipette, cover the membrane area with nuclease-free water.
17. Place ribbon segments of interest onto the nuclease-free water droplet and, if necessary, arrange them using the brush. Let the paraffin and the sliced sample expand for proper microscopic visualization for a couple of minutes. Rapidly, discard the nuclease free water droplet in a fluid motion leaving only the paraffin ribbon segments on the membrane (*see* Note 9).
18. Place LCM slides back onto the hotplate and cover them to reduce the risk of contamination. Keep the slides on the hotplate until completely dry (*see* Note 10).

19. Prepare two staining dishes filled with fresh Xylol (or Histoclear™) under a fume hood. Remove wax from the prepared slides by two consecutive washes of 15 minutes each. Let the slides dry under the hood for another 15 -20 minutes and proceed to the LCM immediately (*see* Note 11).

3.4 Laser-assisted microdissection (LAM) on the laser dissection microsope (LCM)

1. Remove dust and small particles from the LCM using the compressed air spray.
2. Turn on the devices for the LCM (computer, microscope, laser control box) and start the MMI software (*see* Note 12).
3. Calibrate objectives to be used and laser settings, refer to the manufacturer's instructions for details.
4. Cover the ready to laser LCM slides by placing a nuclease-free glass microscopy slide on the flat surface with the PET membrane containing the sectioned samples. Place the assembled slides on the microscope with the microscopy slide facing down and the LCM slide facing up.
5. Equip the cap holder with a collection cap and position it on the magnetic lift mechanism of the microscope.
6. Select the 4X Objective. On the MMI software make an overview scan of the slide.
7. On low magnification (4x) identify embryos on the slide and define location pins; this will allow you to move back to the exact position for the following cutting step.
8. Change to a higher magnification (10-20 x) and go back to the previously defined and pinned embryo locations (Figure 1, A-F).
9. Lower the collection cap, making sure that it makes contact on an empty space with the target embryonic tissue; this will ensure proper adherence of the tissue to the cap. Do not collect tissue over previously collected tissue as this will cause detaching from the collection cap. Use the MMI software tools to draw the desired shape for the laser to cut (*see* Note 13) (Figure 1, G-I).
10. Press the cut function, observe and control that the laser precisely cuts the area of interest.
11. Raise the collection cap and make sure that the embryo slice has been collected (*see* Note 14) (Figure 1, J-L).
12. Repeat from step 8 until all embryos are collected, for each defined pin on a given LCM slide.
13. Continue with the rest of the LCM slides prepared for a given session. Repeat from step 4 until all prepared LCM slides are processed (*see* Note 15).
14. Select the 4X Objective. Examine the collection cap for an overview of the collection and to identify possible dirt; if dirt is found, remove it (*see* Note 16).
15. Close the cap and store at -80°C until RNA extraction (*see* Note 17).

3.5 RNA extraction, cDNA synthesis, and library preparation for Illumina RNA sequencing

1. Extract RNA using a kit designed to optimize recovery from low input samples. We have been successful in the described systems with a minimum of 50 slices of embryos. We routinely use Arcturus™ PicoPure™ RNA isolation kit (Applied Biosystems by Thermo Fisher Scientific, USA) with consistently good results. Follow the manufacturer's instructions (*see* Note 18).

2. Validate RNA extraction by running Agilent 2100 Bioanalyzer Plant RNA Pico Chip, representative traces of picogram quantities of RNA can be detected (Figure 2A) (*but see* Note 19).
3. Amplify cDNA from the extracted RNA using as a baseline the Smart-Seq2 protocol *(34)*. We routinely use the SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing (Clontech Laboratories Inc., USA) with consistently good results. Follow the manufacturer's instructions.
4. Validate cDNA amplification output and quality by running Agilent 2100 Bioanalyzer High sensitivity DNA Chip (Figure 2B) or an Agilent High Sensitivity D1000 ScreenTape.
5. Prepare libraries for sequencing using the Nextera® XT (Illumina Inc., USA) library preparation kit. Follow the manufacturer's instructions.
6. Validate library output and quality by running Agilent 2100 Bioanalyzer High sensitivity DNA Chip (Figure 2C) or an Agilent High Sensitivity D1000 ScreenTape.
7. Sequence your transcriptomic libraries on an Illumina Sequencing machine. We have obtained good quality sequencing data on the Illumina HiSeq2500 and Illumina HiSeq4000 platforms.

Notes

1. It is critical to maintain the samples from collection upon embedding at < 4°C to avoid RNA degradation or modification of gene expression patterns. Immerse in fixation solution rapidly, use ice-cold solutions, and do not allow temperatures to rise.
2. For our studies, we have focused on globular stage embryos. Manual pollinations are performed and collection is done upon reaching the desired stage. Earlier or later stages should be assessed depending on the system. For wild tomatoes, this stage is reached 15 days after pollination (DAP). For *Boechera holboelli* it is reached at 7 DAP under our growth condition.
3. In the case of *Boechera*, depending on the species and stage targeted, peeling off the valves can be quicker and less prone to damaging the ovules harboring the embryos than cutting them open. For peeling, the silique is placed on a glass slide, held at the base with one pair of forceps while another pair of forceps is used to peel off one valve at a time.
4. Work fast to avoid drying of the tissue as this will impede the impregnation with paraffin in the following embedding step.
5. Work fast to avoid solidification of paraffin wax around samples as this will impede proper placement of the samples in the paraffin block and will interfere with proper sectioning.
6. Proper positioning of the samples to maximize embryo recovery is critical for obtaining good amounts of embryo slices upon LAM, and for optimizing the efficiency of the protocol. The position on the paraffin block will vary according to the plant system used. For wild tomatoes, the exposed part of the fruit halves should be as close as possible and facing the bottom of the paraffin block. Likewise, for *Boechera* siliques, these should be as close as possible and directly facing the bottom of the paraffin block. Multiple wild tomato fruits or siliques can be placed close together on a paraffin block to optimize output of embryo slices per block processed.
7. Proper trimming of the paraffin block is critical to maximize efficiency of LAM with regard to number of embryo slices recovered per LCM slide. Leave a maximum of 1 to 2 mm edges of paraffin around the sample, in order to be able to stack as many slices of sample as possible in a stretch of the paraffin ribbon. Accurate

parallelism between the edges of the paraffin block will ensure straight paraffin ribbons that will allow you to put more ribbons onto a single LCM slide.

8. Section thickness needs to be adjusted depending on the plant system. Thinner sections will give better structural resolution but less RNA quantity. In our systems, embryo sections of 8 µm have yielded good amounts of high quality RNA while still maintaining good structural resolution (Figure 1).
9. To ensure good RNA quality, limit the time the paraffin ribbons remain exposed to heated nuclease-free water while expanding on the heating plate. Alternatively, a methanol-water mixture can be used in this step to increase RNA quality but the surface tension will be lower, which affects spreading of the paraffin ribbons.
10. To ensure good RNA quality, limit the time the LCM slides remain on the heating plate to the exact time necessary for them to be completely dry. Two hours is usually enough for our samples. It is important that samples are completely dry as this directly impacts on RNA quality and structural resolution.
11. After dewaxing your samples, tissue and RNA is completely exposed; therefore work quickly to limit the time of exposure. LCM slides that will not be immediately processed can be placed on a heating plate at 42 °C for up to a few hours. This will prevent any humidity from compromising RNA quality until further processing. Long term storage and posterior use of dewaxed LCM slides is not recommended.
12. This protocol is described based on the technology of collecting laser-cut slices on a cap making use of electrostatic forces and should remain more or less the same across similar devices. Details might vary between different versions of the software and equipment. Consult user handbooks and manufacturer's instructions for instructions particular on instrument and software usage.
13. Draw the cutting line slightly on the inner side of the target embryonic tissue (Figure 1, G-I). This ensures that no endosperm tissue will be collected and contaminate your sample.
14. If the embryonic tissue was not dissected at once, sectioning needs to be repeated. Lower the cap again and repeat the sectioning. Increasing laser intensity or setting the software to automatically do two repeats of sectioning will ensure proper dissection.
15. Do not work for more than five hours on a given collecting cap to reduce time of exposure of collected tissue and its RNA to room temperature. Store the collection cap at -80°C and start with a new collection cap. Do not overload collection caps with samples and make sure each slice of embryonic tissue collected is attached to the collection cap. If a collection cap is filled up, store it at -80 °C and continue dissecting on a new collection cap.
16. Often, dust and fibers find their way onto the collection cap. If this is the case, try to manually remove them using a small pipette tip. Sometimes, lack of accuracy when drawing the laser path or improper calibration of the objectives may lead to a few unspecific cells being captured; in this case, use the ablation tool to destroy these unspecific cells in order to ensure the specificity of the transcriptomes to be produced.
17. Minimize the time between LAM and RNA extraction. Best results are obtained when RNA extraction and cDNA synthesis are performed immediately after LAM. Tissue dissected by the LCM can be stored for a couple of weeks and it will still produce good data for transcriptomic analyses.
18. If several caps were collected from the same replicate/treatment, it is possible to pool extracts from several collection caps and collect all RNA in a single extraction column; this will increase RNA output. We recommend a minimum of 50 slices of embryos for successful data production.

19. We have experienced that often RNA extracts of very low quantity do not resolve well on Agilent 2100 Bioanalyzer Plant RNA Pico Chips. Nonetheless, we have been able to amplify cDNA and obtained good quality transcriptomic data from RNAs that did not resolved well on Bioanalyzer Plant RNA Pico Chips.

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Figures

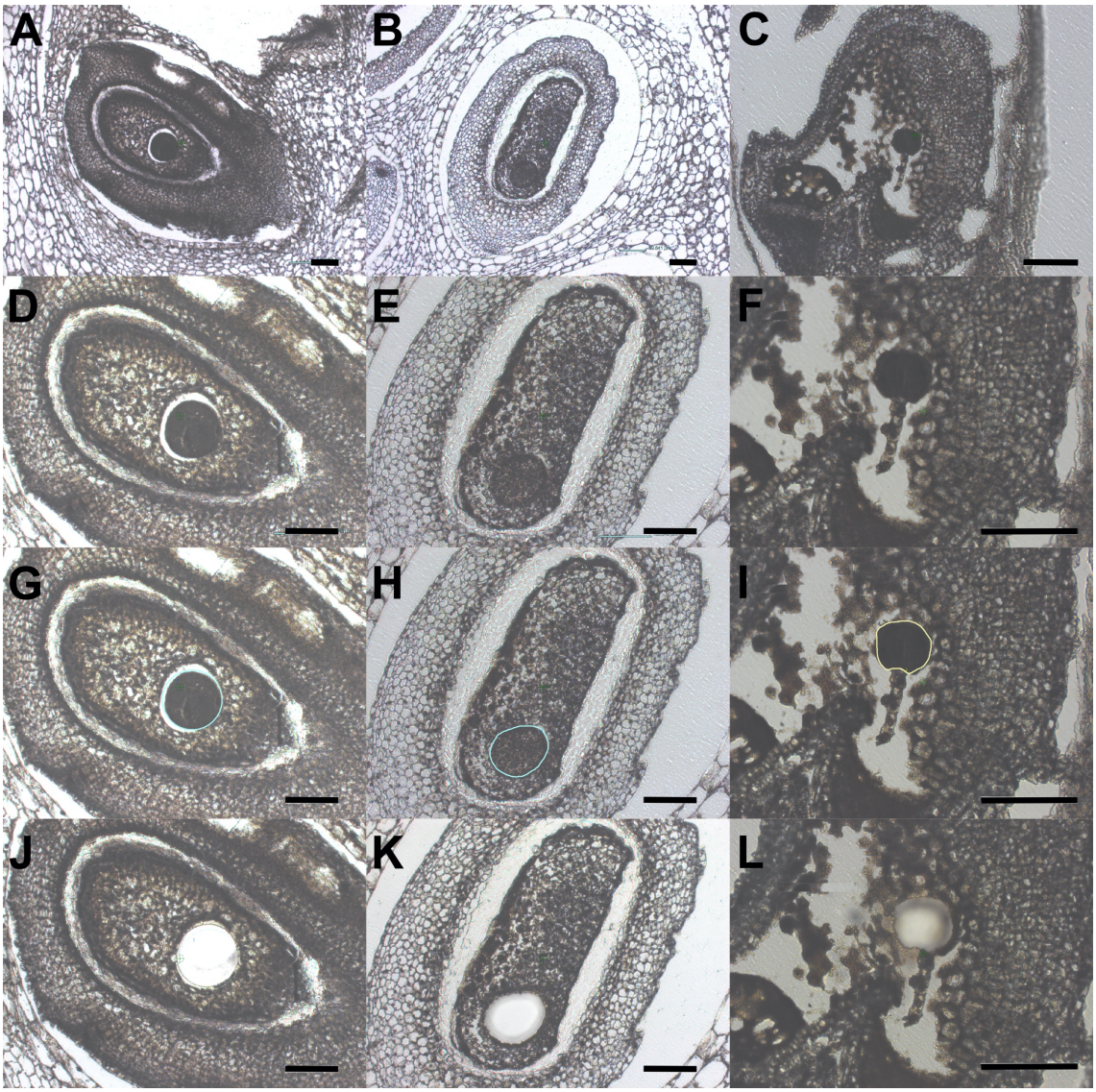

Figure 1. Microscopic observations of embryos under the laser capture microscope (LCM). (A, D, G and J) *Solanum peruvianum*, (B, E, H and K) *Solanum chilense*, (C, F, I and L) *Boechera holboelli*. Embryos are clearly discernable in dry, thin-sections of seeds (A-C) and their outlines nicely demarcated at higher magnification (D-E). Accurate drawing of the embryo boundaries for laser cutting secures a high specificity of transcriptomic data (G-H). Embryonic sections are precisely excised after laser-assisted microdissection (LAM) (J-L).

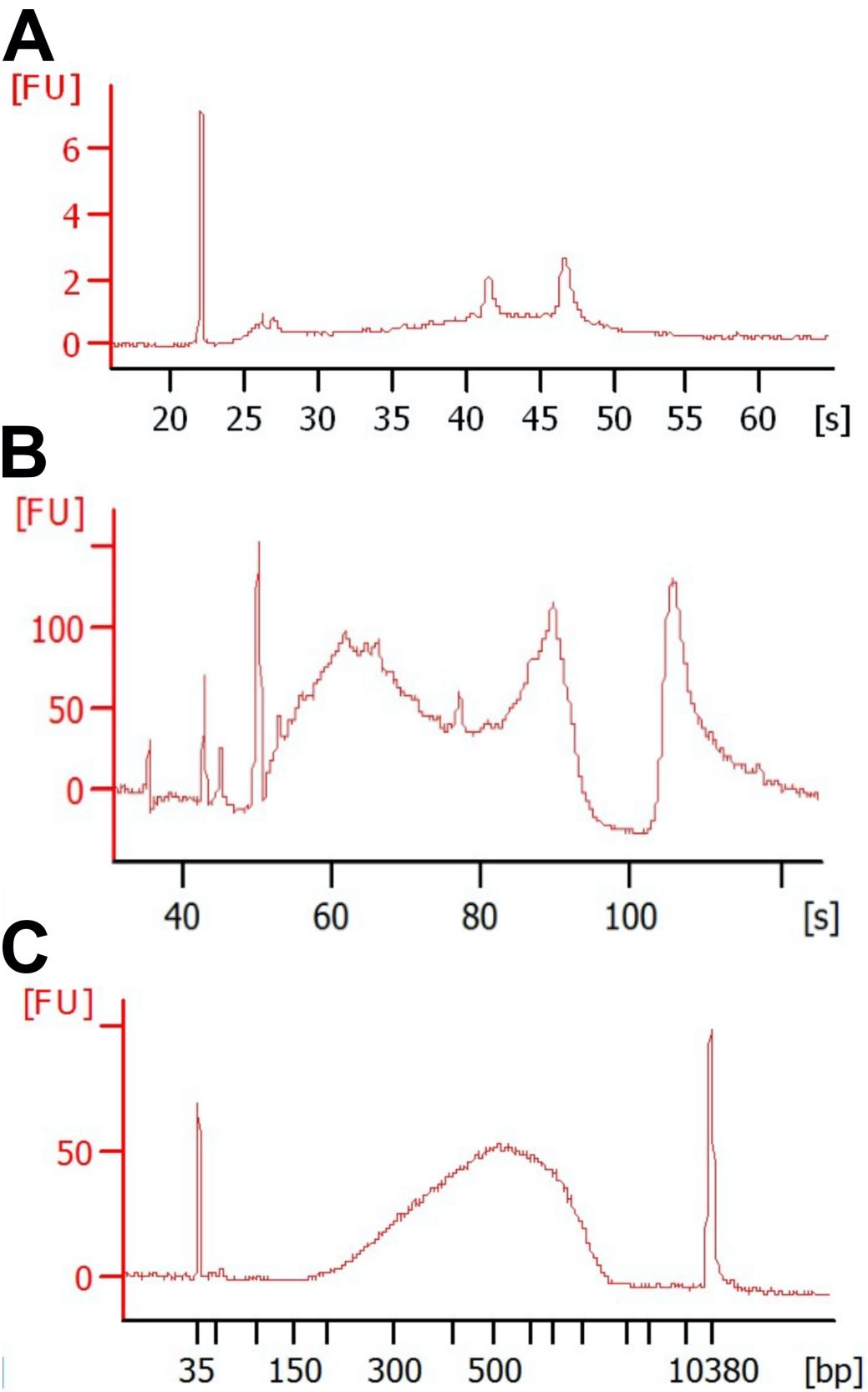

Figure 2. Quality control through Agilent 2100 Bioanalyzer assays. **(A)** Representative trace of 47 pg LAM RNA in a Plant RNA Pico Chip. **(B)** Trace of amplified cDNA from *B. holboelli* embryo RNA run in a High sensitivity DNA Chip. **(C)** Trace of a *B. holboelli* embryo Nextera XT library run in a High sensitivity DNA Chip.